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QUANTITATIVE RT-PCR ANALYSIS OF *PHYTOPHTHORA* SPECIFIC GENES EXPRESSED DURING PHYTOPHTHORA *CAPSICI–PIPER COLUBRINUM* INTERACTION

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ABSTRACT

The genus *Phytophthora*, contains destructive plant pathogens that cause enormous economic damage to many important crop species. These funguses like oomycete pathogens employ diverse mechanisms to break down plant defence mechanism. An understanding of the process by which these pathogens colonize their host is essential to develop appropriate disease management strategies. *Phytophthora capsici* which causes foot rot, is a serious disease in black pepper growing areas throughout the world and *P.colubrinum*, a distantly related species is highly resistant to the pathogen infection. The genes selected in this particular study, are responsible for the production of pathogen-derived molecules which play an important role in the process of colonization of the pathogen on the particular host, as well as in modulating defence responses in the particular host. Quantitative RT-PCR was employed to assess the level of expression of some of the genes of *P.capsici* like Glycoside hydrolase, NPP 1, RXLR and Pectatelyase, found to be expressed during *P.capsici-P.colubrinum* interaction. Glycoside hydrolase and RXLR genes showed high levels of expression during early stages of infection (up to 16 hpi), whereas the NPP1 gene showed maximum expression at later stages of infection (at 72 hpi). Pectatelyase gene showed high level of expression at early stages of infection but was then down regulated during the later stages of infection. The expression of these genes during initial phase of infection gives an idea about the importance of these genes during host colonization and in defence response of the plant to the particular pathogen.

KEYWORDS: Glycoside Hydrolase, NPP1, RXLR, Pectate Lyase, *Phytophthora capsici*, *Piper colubrinum*

INTRODUCTION

Oomycetes, also known as water molds, are among the organisms that cause many serious plant diseases and large economic losses in agriculture the major plant-pathogenic genera of oomycetes are *Albugo*, *Bremia*, *Peronospora*, *Phytophthora*, *Plasmopara* and *Pythium* (Kamoun et al., 2003). Among these genera, species of the genus *Phytophthora* are arguably the most destructive pathogens of dicotyledonous plants like potato, pepper, tomato, soybean, pea and alfalfa (Kamoun et al., 2003; Tyler et al., 2006).

These filamentous pathogens penetrate plant tissue and extend hyphae into the spaces between cells in search of nutrients. As hyphae extend through plant tissue, they can form intimate contacts with host cells by developing highly specialized structures known as haustoria (Panstruga and Dodds, 2009; Dodds and Rathjen, 2010). These structures are a major interface for exchange of important chemicals between pathogen and host during infection, including the delivery of pathogen-derived molecules that are essential for infection.

These molecules, collectively known as elicitors or effectors, are thought to be major determinants of pathogenicity, and understanding their molecular functions has become a major theme in the study of plant–microbe

interactions. However, our understanding of how filamentous plant pathogen effectors modulate plant immunity is rudimentary. Effectors manipulate plant processes to the advantage of the pathogen, promoting host infection and colonization and they also activate plant immune receptors on resistant hosts (Win et al 2012).

Black pepper, (*Piper nigrum* L.), the king of spices is an important commercial crop with considerable export earnings to the country. *Phytophthora* foot rot has been identified as a major production constraint not only in India but also in other parts of the world. Foot rot is caused by *Phytophthora capsici*, a soil borne oomycete pathogen. *Piper colubrinum* a wild species of piper is known to be resistant to foot rot disease. The present investigation mainly focuses on the expression analysis of genes like glycoside hydrolase, NPP1, RXLR and pectatelyase expressed during *P. capsici-P. colubrinum* interaction.

To breach the cell wall barrier and cause complete disintegration most pathogenic oomycetes have taken advantage of a complex assortment of cell wall-degrading enzymes (CWDE) of which glycoside hydrolases (GH) are very important. Glycoside hydrolases (GH) causes the hydrolysis of the glycosidic bonds that form cellulose. Few reports indicate that CWDE-coding genes like glycoside hydrolase exist in its *P.infestans* genome (Constanzo et al 2006). NPPs, namely necrosis-inducing *Phytophthora* proteins, also called NLPs (Nep1- like proteins), because the protein shares substantial sequence similarity with the first discovered member (named Nep1), was defined as the necrosis and ethylene-inducing protein isolated from *Fusarium oxysporm* f.sp. *erythroloxyli* in liquid cultures (Bailey, 1995). NPPs represent a new class of necrotic elicitors. Genes encoding NPPs, or the proteins themselves, have been detected in superfamilies in eukaryotic and prokaryotic organisms. The NPPs are not found in plants, animals, but they often occur in fungi, bacteria and also in oomycetes. Oomycetes, particularly *Phytophthora* species like *P. infestans*, *P. parasitica*, *P. aphanidermatum*, *P. sojea*, and *P. ramorum* form NPPs (Pemberton and Salmond, 2004). Although NPPs are present in phylogenetically distant organisms, the NPP sequence has remained a remarkably conserved domain called the necrosis-inducing *Phytophthora* protein 1 (NPP1) (Fellbrich et al., 2002). A hepta-peptide (GHRHDWE) and some conserved cysteine residues are present in the NPP1 sequences isolated from various organisms. Based on the number and position of cysteine residues, NPPs are classified into two groups (Gijzen and Nurnberger, 2006). It is known to induce defence response in *Arabidiopsis*. NPP 1 structural homologs are found in fungi, oomycetes and bacteria but not in plants (Fellbrich et al 2002).

RXLR effectors of pathogenic oomycetes appear to be modular proteins with two main functional domains. The N-terminal domain has a signal peptide and conserved RXLR region that functions in secretion and targeting and; the remaining C-terminal domain carries the effector activity and operates inside plant cells (Kamoun 2006). Pectate lyases (PELs), which cleave glycosidic bonds of pectate or low methylated pectin by a trans-eliminative mechanism to yield unsaturated products, are widely distributed among microorganisms, such as *P. capsici* (Herron et al 2000).

MATERIALS AND METHODS

Piper colubrinum plants maintained in green house were used for this study. Generally young leaves were used for pathogen inoculation. *Phytophthora capsici* grown on carrot agar medium was used for inoculation. Isolate 05-06 maintained at the National Repository for *Phytophthora* at Indian Institute of Spices Research were used for the present study. 72 h old culture were used for inoculation. The mycelial discs measuring 10 mm were cut out from the culture plates and used for inoculation. The discs were kept below the surface of the leaves along with wet cotton and held together with cellophane tape. A total of eight treatments (different time intervals) were used for RNA extraction. These time intervals

include 0, 2, 4, 8, 16, 24, 48 and 72 h post inoculation (hpi). The genes selected for this study were based on the sequence information obtained from transcriptome sequencing done on *P. colubrinum* challenge inoculated with *P.capsici* at the Indian Institute of Spices Research (Johnson et al, unpublished results). Primer designing was done using Primer 3 software **(Table 1)**

RNA Extraction

Total RNA was extracted from the inoculated and control samples by Trizol method (Invitrogen, USA). The integrity of the RNA was checked on 1.2% agarose gel. DNase treatment was given to the RNA using DNAse (Fermentas, USA) as per instructions from the manufacturer. The RNA was quantified using Biophotometer plus (Eppendorf, Germany).

cDNA Synthesis

About 2 µg of total RNA was used for cDNA synthesis using the kit (Fermentas, USA). The RNA was first combined with 1µl oligodT_{18} primer and heated in a PCR block at 65°C for 5 min and quickly chilled for about 5 min. To this mixture 4 µl of 5X cDNA synthesis buffer, 2 µlof 10mM dNTP, 1 µl of Ribolock RNase inhibitor and 1 µl of Revert-aid MMLV Reverse transcriptase (200 units). The mixture was subjected to reverse transcription at 42°C for 60 min and incubation at 70°C for 15 min. The cDNA was stored at -20°C till further use.

Real Time PCR

The relative expression levels of the selected genes were determined at various post-inoculation times by real time PCR. About 100 ng of cDNA , 0.8µM each of forward and reverse primers, 12.5µl of 2X QuantiFast SYBR Green PCR master mix (Qiagen, Germany) were used for each of the three replicates. The real time PCR conditions included initial activation step at 95°C for 5 min, denaturation at 95°C for 20 sec, annealing at 58°C for 20 sec and extension at 72°C for 20 sec for 35 cycles. Threshold cycle (C_T) value for each reaction and the relative expression values were calculated using software version 2.0.2 Rotor gene –Q (Qiagen, Germany). The PCR signals were normalized to that of Actin gene. The cDNA from 72 h old mycelial RNA was used as control.

RESULTS

The specificity of the designed primers for the amplification of the different genes was checked by PCR and the products were run on agarose gels in which they gave single bands of expected length. The expression levels of Glycoside hydrolase, NPP 1, RXLR and Pectate Lyase genes during the interaction between Piper *colubrinum* and *Phytophthora capsici* at different time points were quantified using Real time PCR. Glycoside hydrolase showed higher expression at 16 hpi and was further down regulated from 24-72 h (**Figure 1).** NPP 1 gene showed high level of expression at later stages of infection mainly at 72 hpi, about 118 fold when compared to the control **(Figure 2).** RXLR gene showed an early induction and high expression about 5.26 fold at 2 hpi **(Figure 3).** Pectate lyase gene showed high level of expression ranging from 100 fold to about 450 fold from 2hpi to 24 hpi and was further down regulated at 48 and 72 hpi **(Figure 4).**

DISCUSSIONS

All the genes selected in this particular experiment showed distinctly different expression patterns during the infection time course. Glycoside hydrolase is an important protein which belongs to the cell wall degrading enzyme (CWDE) group. Here glycoside hydrolase shows high expression at early stages of infection (almost 60 fold) at 16 hpi.

Similar results by Kebdani et al. (2010) showed an early expression of cell wall degrading enzymes based on qRT-PCR conducted in *Phytophthora parasitica*. Cell wall degrading enzymes were particularly abundant among the transcripts transiently accumulating during the first few hours of infection (Judelson et al. 2008).

NPP1 showed high level of expression at late stages of infection ie, at 72 hpi (about 118 fold) during *P.capsici*-*P.colubrinum* interaction. Expression analyses of the *Phytophthora infestans NPP1* gene showed that it is up-regulated during late stages of colonization of tomato (Kanneganti 2006), especially during necrotic stage of infection (Qutob 2002). In case of *P.capsici*-tomato interaction the biotrophic stage occurs in the first 24 hpi and later the necrotrophic stage starts from 24 hpi onwards (Jupe 2013).

The high levels of expression of RXLR effector at early stages of infection at 2 hpi coincides with the biotrophic phase of infection. Similarly in *P.infestans* RXLR effectors were transcriptionally induced during early stages of infection of potato (Whisson *et al.*, 2007). At least 79 of the predicted *P. infestans* RXLR effectors, including RXLR effectors with known avirulence activity, such as *Avr3a*, *Avr4* and *AVRblb1*/*ipiO*, showed a distinct expression pattern with a sharp peak of transcriptional up-regulation during the biotrophic phase of the infection (Haas *et al.*, 2009).

CONCLUSIONS

Pectatelyase showed high level of expression up to 24 hpi and later its expression was down regulated from 48 hpi onwards. This might probably be due to the pathogen colonization of the plant at early stages and the reduction in expression at later stages was mainly due to the high level of expression of defence proteins like β-1,3 glucanase and osmotin at 72 hpi by the plant in *P.capsici* inoculated *P.colubrinum* (Vijesh Kumar et al, unpublished results), which might be suppressing the pathogen mediated protein expression or causing the death of the pathogenic organism.

The present study provides insight into the expression pattern of genes responsible for producing pathogen derived molecules during various phases of *P.capsici* infection in *P.colubrinum*.

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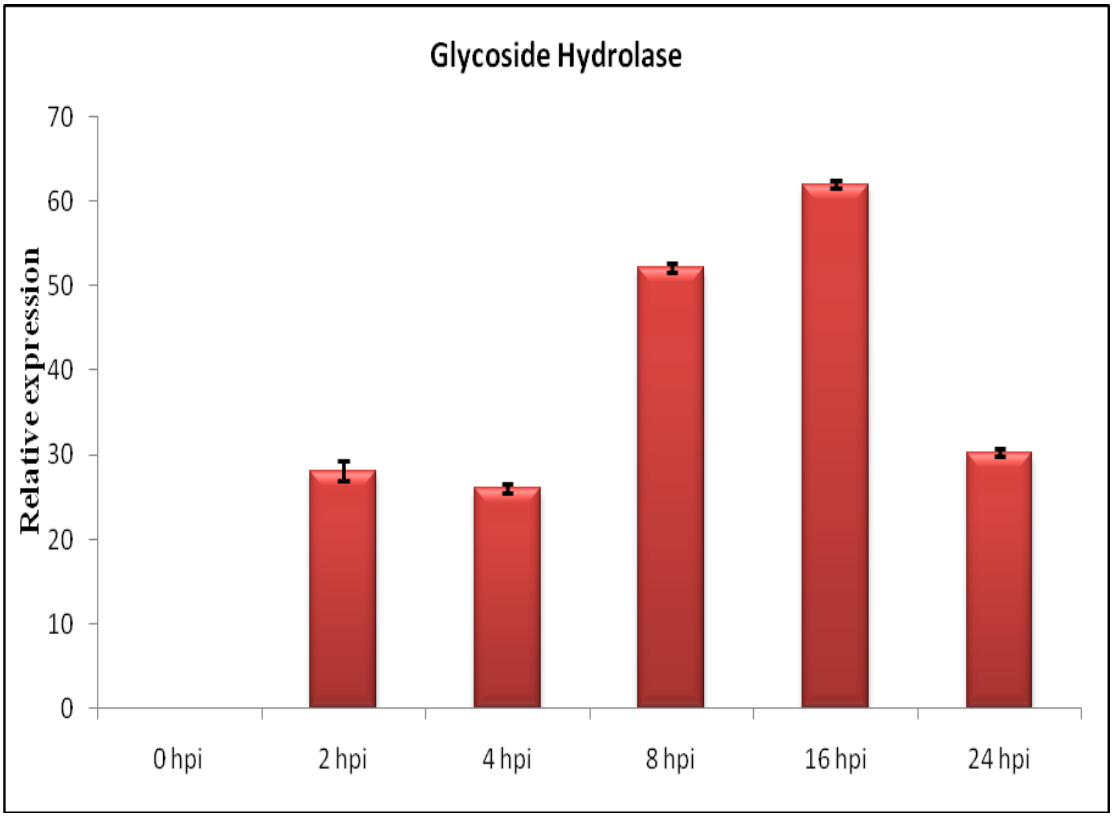

Figure 1: Relative Expression of Glycoside Hydrolase Gene

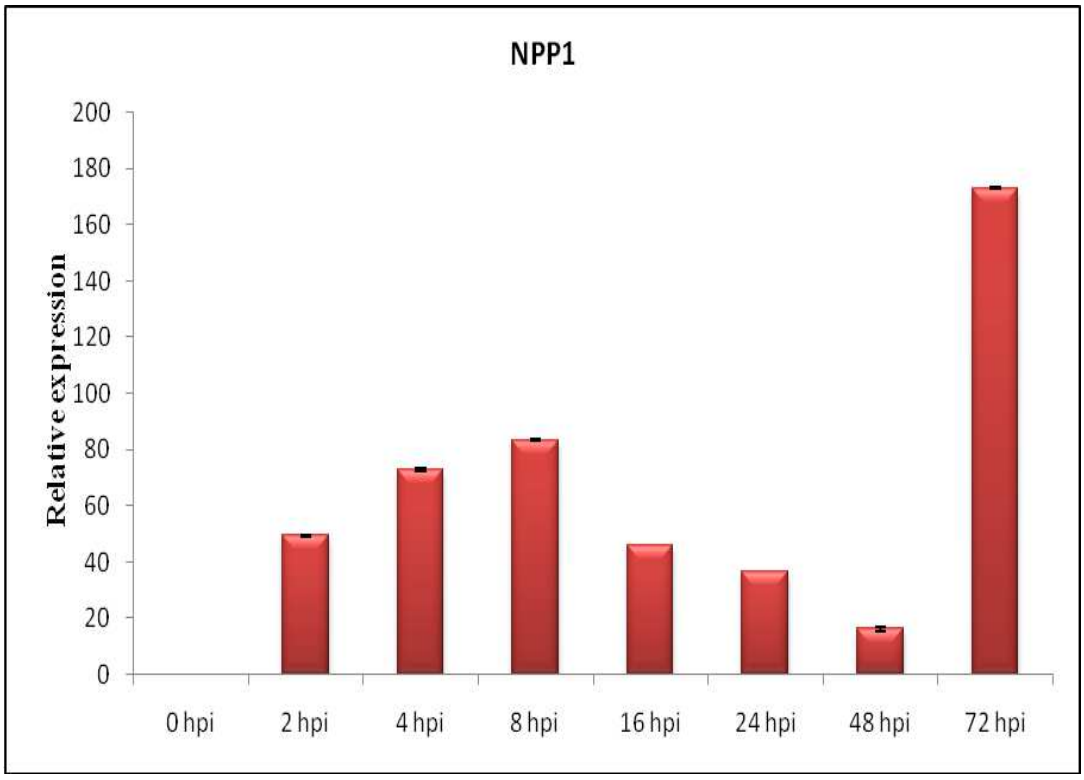

Figure 2: Relative Expression of NPP1 Gene

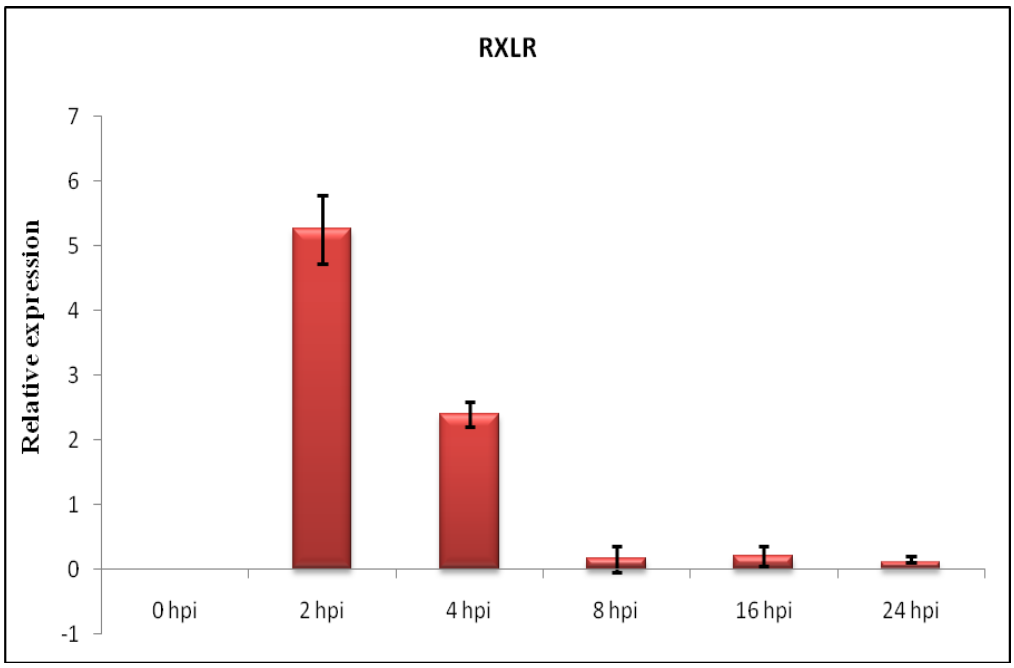

Figure 3: Relative Expression of RXLR Gene

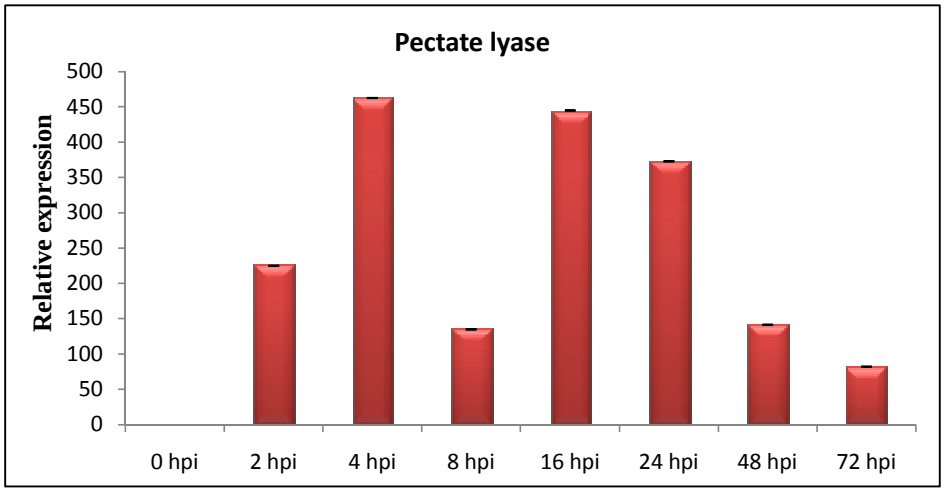

Figure 4: Relative Expression of Pectate Lyase Gene

Table 1: List of Primer Sequences Used for the Present Study

Sl No	Gene	Primer Sequences		Amplicon Length(bp)
1	Actin	CACATCTGCTGGAAGGTGCT	F	143
		CACTACTGCAGAGCGGGAA	R	
2	Glycoside Hydrolase	CAGTGTGTCGGTGAACGGTA	F	134
		TCTTGGAGCGGTCAATCACC	R	
3	NPP 1	GATGTGTGCCTTACCCTGCT	F	126
		ATGTGGAACGGCCGTAAACT	R	
4	RXLR	AGAGGGGATGGCTGTCAGAT	F	134
		GCCCTTGTCGAAGATCGTGT	R	
5	Pectate Lyase	ACGGTCAAGATCAACGGCTT	F	163
		TGGCCTGGTCGTTGTAGTTC	R	